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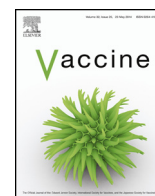
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# Haematopoietic depletion in vaccine-induced neonatal pancytopenia depends on both the titre and specificity of alloantibody and levels of MHC I expression



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## ABSTRACT

Bovine Neonatal Pancytopenia (BNP) is a disease of calves characterised by haematopoietic depletion, mediated by ingestion of alloantibodies in colostrum. It has been linked epidemiologically to vaccination of the dams of affected calves with a particular vaccine (Pregsure) containing a novel adjuvant. Evidence suggests that BNP-alloantibodies are directed against MHC I molecules, induced by contaminant bovine cellular material from Madin-Darby Bovine Kidney (MDBK) cells used in the vaccine's production. We aimed to investigate the specificity of BNP-alloantibody for bovine MHC I alleles, particularly those expressed by MDBK cells, and whether depletion of particular cell types is due to differential MHC I expression levels.

A complement-mediated cytotoxicity assay was used to assess functional serum alloantibody titres in BNP-dams, Pregsure-vaccinated dams with healthy calves, cows vaccinated with an alternative product and unvaccinated controls. Alloantibody specificity was investigated using transfected mouse lines expressing the individual MHC I alleles identified from MDBK cells and MHC I-defined bovine leukocyte lines. All BNP-dams and 50% of Pregsure-vaccinated cows were shown to have MDBK-MHC I specific alloantibodies, which cross-reacted to varying degrees with other MHC I genotypes. MHC I expression levels on different blood cell types, assessed by flow cytometry, were found to correlate with levels of alloantibody-mediated damage *in vitro* and *in vivo*. Alloantibody-killed bone marrow cells were shown to express higher levels of MHC I than undamaged cells.

The results provide evidence that MHC I-specific alloantibodies play a dominant role in the pathogenesis of BNP. Haematopoietic depletion was shown to be dependent on the titre and specificity of alloantibody produced by individual cows and the density of surface MHC I expression by different cell types. Collectively, the results support the hypothesis that MHC I molecules originating from MDBK cells used in vaccine production, coupled with a powerful adjuvant, are responsible for the generation of pathogenic alloantibodies.

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## 1. Introduction

Bovine Neonatal Pancytopenia (BNP) is a disease of calves that emerged in Europe from 2007 [1,2] and subsequently in New Zealand [3]. It is characterised by profound depletion of peripheral leukocytes and thrombocytes and bone marrow trilineage

hypoplasia, and is mediated by the ingestion of alloantibodies in colostrum [4–6]. BNP has been linked epidemiologically to vaccination of the dams of affected calves with a particular inactivated Bovine Viral Diarrhoea Virus (BVDV) vaccine (Pregsure BVD, Pfizer Animal Health) which incorporates a novel adjuvant formulation [7–9]. However the incidence of clinical BNP has been reported to be only 0.0016% per dose of vaccine sold [10]. This, together with variable alloantibody binding of individual sera to lymphocytes from different donor cows [11], suggests specificity for a highly polymorphic antigen.

Alloantibodies in BNP-dams have been shown to recognise Major Histocompatibility Complex class I (MHC I) molecules [12–14], leading to the hypothesis that Pregsure contains bovine

**Abbreviations:** BNP, Bovine neonatal pancytopenia; BVDV, Bovine Viral Diarrhoea Virus; CDC assay, complement-dependent cytotoxicity assay; MDBK cells, Madin-Darby Bovine Kidney cells; PRA, Panel Reactive Antibody.

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MHC I originating from the Madin-Darby Bovine Kidney (MDBK) cells used in production of the vaccine, and that this is responsible for the generation of alloantibodies in particular cows to which the product is administered. However, the specificity of these alloantibodies for individual MHC I alleles, or quantitative analysis of their functional activity, has not been previously demonstrated. Furthermore it has been suggested that Pregsure might also boost normal pregnancy-induced alloantibodies against paternally-derived MHC antigens [6], which have been detected previously in the sera of healthy cows [15,16].

Evidence for the mechanism of cellular destruction initiated by BNP alloantibody is limited. While one study [11] demonstrated a modest increase in cytophagocytosis following overnight incubation with BNP-sera, cell destruction *in vivo* is more rapid and profound [6] suggesting involvement of complement-mediated lysis. However, previous attempts at inducing complement-mediated lysis by binding of BNP-alloantibody have been reported to be unsuccessful [17]. Complement-dependent cytotoxicity (CDC) assays are routinely used in pre-transplant screening of recipients for MHC I alloantibody, as this method is considered to give clinically relevant results [18,19] and CDC assays have also been used in cattle to detect naturally-occurring pregnancy-induced serum alloantibodies [16,20].

Cellular damage mediated by BNP-alloantibody appears not only to be limited to the haematopoietic system but also to show variable damage to different haematopoietic lineages [6]. This specificity, despite the widespread expression of MHC I by most cells, has not yet been explained. However we and others [6,13] have suggested that this may reflect differences in the level of expression of MHC I by different cell types, as haematopoietic cells of the immune system are known to express high levels of MHC while some tissue cells express very low levels and non-nucleated erythrocytes show no expression [21–23].

This study aimed to use a functional assay to assess BNP-alloantibody specificity for bovine MHC I alleles, in particular those expressed by the MDBK cell line. Furthermore, it aimed to investigate whether the specificity of BNP-pathology for particular cell types is due to the levels of MHC I expressed by the affected cells.

## 2. Methods

### 2.1. Cows

Sera were collected from four groups of cows, (1) Pregsure-vaccinated cows that had produced a calf with clinical signs of BNP confirmed by bone marrow histopathology ( $n = 10$ ) or haematology ( $n = 1$ ) (BNP-dams), (2) Pregsure-vaccinated cows with no history of producing a calf with clinical signs of BNP (Pregsure-cows,  $n = 8$ ), (3) cows vaccinated with an alternative brand of inactivated BVDV vaccine (Bovilis BVD, MSD Animal Health) (Bovilis-cows,  $n = 7$ ), (4) BVDV-unvaccinated, BVDV-free cows (control cows,  $n = 8$ ).

### 2.2. Preparation of cells

Whole blood was collected in EDTA and peripheral blood mononuclear cells (PBMC) prepared by density gradient centrifugation and whole leukocytes by erythrocyte lysis, according to standard methods. Thrombocyte-rich plasma was prepared by centrifugation at 170 g for 10 min. Bovine bone marrow cells were collected from transversely sectioned ribs from a freshly euthanized animal by flushing with media plus 5 mM EDTA using a 7G 100 mm biopsy needle inserted longitudinally into the articular surface. Collected flow-through was centrifuged (400g, 10 min), contaminating erythrocytes lysed prior to washing, and cells stored at

–180 °C in foetal calf serum (FCS) containing 10% DMSO until use.

### 2.3. Production of MHC I defined transformed bovine leukocyte cell lines

Bovine PBMC were transformed by infection with the protozoan parasites *Theileria parva* or *Theileria annulata*, according to published methods [24], to establish continuously dividing cell lines which could be maintained indefinitely in culture [25]. Lines were generated from four cross-bred BNP-dams, and twelve Holstein animals expressing previously defined MHC I haplotypes [26] (Fig. S1), confirmed by both allele-specific PCR [27] and sequencing.

### 2.4. Complement-dependent cytotoxicity assay

A semi-automated complement-dependent cytotoxicity (CDC) assay was established, allowing quantification of live and dead cells following addition of test sera [15]. Cells were stained with 25 µg/ml Hoechst 33342 (Invitrogen) (30 min) and incubated with serial dilutions of sera (30 min) followed by the addition of rabbit complement-HLA-ABC (Invitrogen) (1 h) (all RT, protected from light). The vital nucleic acid stain Sytox Orange, which penetrates cells with compromised plasma membranes but will not cross uncompromised cell membranes, was added at 5 µM (final) and incubated (10 min) prior to automated reading of results by two-channel fluorescence microscopy (Cell Observer, Carl Zeiss, x5, ZEN 2012 software). Positive (pan-MHC I-specific mAb; IL-A88; [28]) and negative (FCS) controls were included with each serum-cell combination, together with a complement control (no serum) and live cell control (no serum and heat-inactivated complement). Total cells (~4000/sample) and dead cells were counted using a macro in Image-J software [29].

To compare results from each cow, the serum dilution giving 80% cell killing was estimated by interpolating between the serum dilutions either side of 80%, assuming data followed a sigmoid curve (Microsoft Excel, 2010). The cut-off of 80% was selected in line with MHC I typing assays employing CDC, where 80–100% is classified as a strong positive [20,30]; 80% cell killing is also equivalent to the reduction in PBMC seen in experimentally induced BNP [6].

For each cow the percentage of positive reactions against the panel of MHC I-defined lines was determined (panel reactive antibody percentage; PRA) (see Fig. 2), as a means of estimating the percentage of calves in the bovine population an individual cow's serum would be expected to react against.

### 2.5. Identification of the MHC I alleles expressed on the MDBK cell line and isolation of full-length gene products

Following isolation of RNA and synthesis of cDNA from MDBK cells, a 375 bp fragment of the MHC I heavy chains, including exons 1 and 2 (which encode the polymorphic  $\alpha 1$  and  $\alpha 2$  domains), was amplified by PCR, using primers that amplify all known classical bovine MHC I alleles, as described previously [31]. Following molecular cloning and sequencing, the sequences were aligned using DNASIS MAX (MiraBio Group, Hitachi Solutions America Ltd.), subjected to an NCBI BLAST search [32] and sequence identities confirmed by comparison with the bovine MHC I EBI database [33]. Full-length cDNAs were isolated using consensus primers complementary to semi-conserved regions at the 5' end of the leader sequence and just beyond the 3' end of the cytoplasmic domain of the MHC gene [31]. The PCR products were cloned and sequenced, allowing identification of the full-length sequence for all but one identified MDBK-MHC I allele. For the remaining allele (BoLA 3\*00402 variant),

alternative primers were designed based on database 3\*00402 sequence data (forward 5'-ACCATGGGCGCGAG-3', reverse 5'-GCCTGAAGCTGGATATAATTG-3') and used to obtain a slightly truncated version of the full-length allele (from 5' leader sequence to the middle of the cytoplasmic domain). The identity of the final product was confirmed by sequencing.

### 2.6. Production of transformed mouse cell lines expressing the individual MDBK-MHC I alleles

Stably transfected P815 lines expressing the individual MDBK-MHC I alleles were produced by transfection of the MHC I heavy chain alleles in pc-DNA-3.1/V5-his TOPO vectors into murine P815 cells by electroporation and culture in selective media (500 µg/ml G418). Further selection of cells expressing the class I products was conducted by indirect immunofluorescence staining with the bovine MHC I-specific mAb IL-A88 and polyclonal FITC-conjugated goat anti-mouse IgG/A/M (Sigma-Aldrich) and single cell sorting (FACSaria III, BD Biosciences) allowing the production of clonal lines. These were expanded and screened for expression by flow cytometry, with the highest expressing clones selected for subsequent work.

### 2.7. Indirect immunofluorescence staining for MHC I expression

Cells were re-suspended at  $2 \times 10^7$  cells/ml in either unsupplemented filtered (0.2 µm) RPMI, for thrombocytes, or RPMI supplemented with 2% FCS, 0.2% NaN<sub>3</sub> for other cell types, and stained by indirect immunofluorescence with mAb IL-A88 as described above. For the washing steps, thrombocytes were centrifuged at 1870 g for 10 min and other cell types at 670 g for 1 min. Cells were analysed by flow cytometry, FACS Calibur or LSR Fortessa (BD Biosciences), and data analysed with FlowJo software (TreeStar Inc.). Where dead cell staining was required, 5 nm Sytox Red (Life Technologies) was added to the cells immediately prior to analysis.

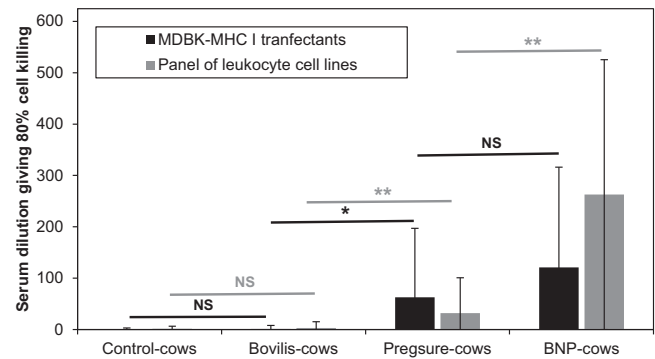
## 3. Results

### 3.1. Identification of MDBK-MHC I alleles

In order to identify the MHC I alleles expressed by MDBK cells, used in production of virus for Pregsure vaccine, cloned PCR products of MHC I heavy chain were subjected to nucleotide sequencing. Five distinct alleles were identified; 3\*01101, 3\*05001, 3\*04801, a variant of 3\*00402 (3\*00402v) which differs from the EBI reference sequence by four amino acids, and a fifth allele, as yet unnamed (accession number DQ121179.1). The alleles 3\*01101, 3\*04801 and 3\*00402 comprise the recently published BF7 haplotype (26), suggesting that the remaining alleles (3\*05001 and DQ121179.1) constitute a novel haplotype. While the existence of additional unidentified MHC I alleles cannot be completely ruled out, sequencing of a large number of clones ( $n = 62$ ) obtained using two sets of primers suggests that this is unlikely.

### 3.2. Cytotoxic activity of cow sera for the MDBK-MHC I alleles

To assess the cytotoxicity of cow sera for the MDBK-MHC I alleles, cow sera were tested against transfected P815 cells expressing the individual MDBK-MHC I alleles using the CDC assay. After subtraction of background cytotoxicity against untransfected P815 cells, Pregsure-vaccinated dams were shown to have significantly higher titres of functional cytotoxic alloantibodies specific for the MDBK-MHC I alleles than cows vaccinated with an alternative BVDV vaccine; however titres were not significantly different



**Fig. 1.** Cows vaccinated with Pregsure have significantly higher titres of functional serum alloantibody against the MDBK-MHC I alleles and against bovine leukocytes. Serum dilutions giving 80% cell killing against the five transfected P815 cells lines expressing the MDBK-MHC I alleles (black bars) and a panel of twelve MHC I defined bovine leukocyte cell lines (grey bars). Mean values  $\pm$  one standard deviation are shown. T-tests, Genstat, \*\*  $p < 0.001$ , \*  $p < 0.01$ , NS  $p > 0.05$ . The reactivity of serum with the transfectants and leukocyte lines are not directly comparable due to differences in levels of MHC I expression and susceptibility to complement lysis between different cell types, and variable sharing of MDBK target epitopes by the MHC I alleles in the bovine lines (see Fig. 2).

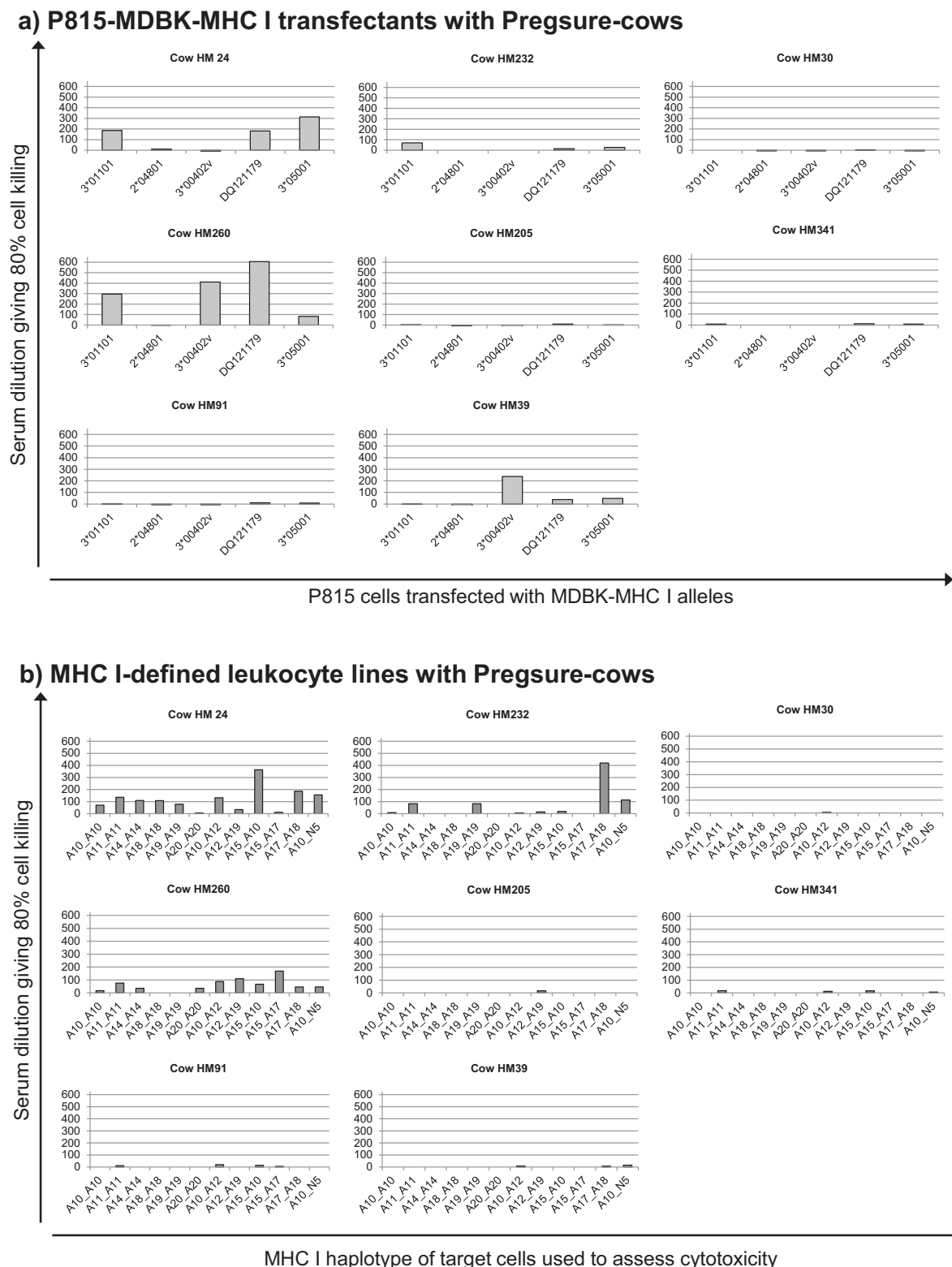
between BNP-dams and Pregsure-vaccinated dams that had produced healthy calves (Fig. 1). The titres and specificities of the alloantibodies for the different MDBK alleles were found to vary between individual Pregsure vaccinated cows (Fig. 2).

### 3.3. Cytotoxicity of cow sera for a panel of MHC I-defined bovine leukocytes

In order to determine the functional activity of BNP-alloantibody against bovine cells of diverse MHC types, sera were tested against a panel of 12 MHC I-defined bovine leukocyte cell lines (Fig. S1) from Holstein cattle, using the CDC assay. In contrast to humans where each MHC I haplotype expresses a fixed complement of three classical MHC I genes, cattle show region configuration polymorphism in the MHC with between one and three genes expressed on any haplotype [34]. Thus these lines express 20 defined bovine MHC I alleles [26] and represent haplotypes expressed by >94% of the Holstein cattle population [35]. Pregsure-vaccinated cows were shown to have significantly higher titres of functional cytotoxic alloantibodies against the panel of cell lines than unvaccinated cows or cows vaccinated with an alternative BVDV vaccine. Pregsure-vaccinated cows which had produced a BNP-affected calf had significantly higher titres than those that had produced a healthy calf (Fig. 1). Individual BNP-dams were found to have cytotoxic alloantibodies of variable titres and specificities against the different cell lines (Fig. 2). CDC assays using autologous cell lines generated from four BNP-dams provided a negative control, with no serum achieving 80% cell killing, at any dilution tested (serum at 1/5 dilution, mean cell killing 19.9% S.D. 8.4).

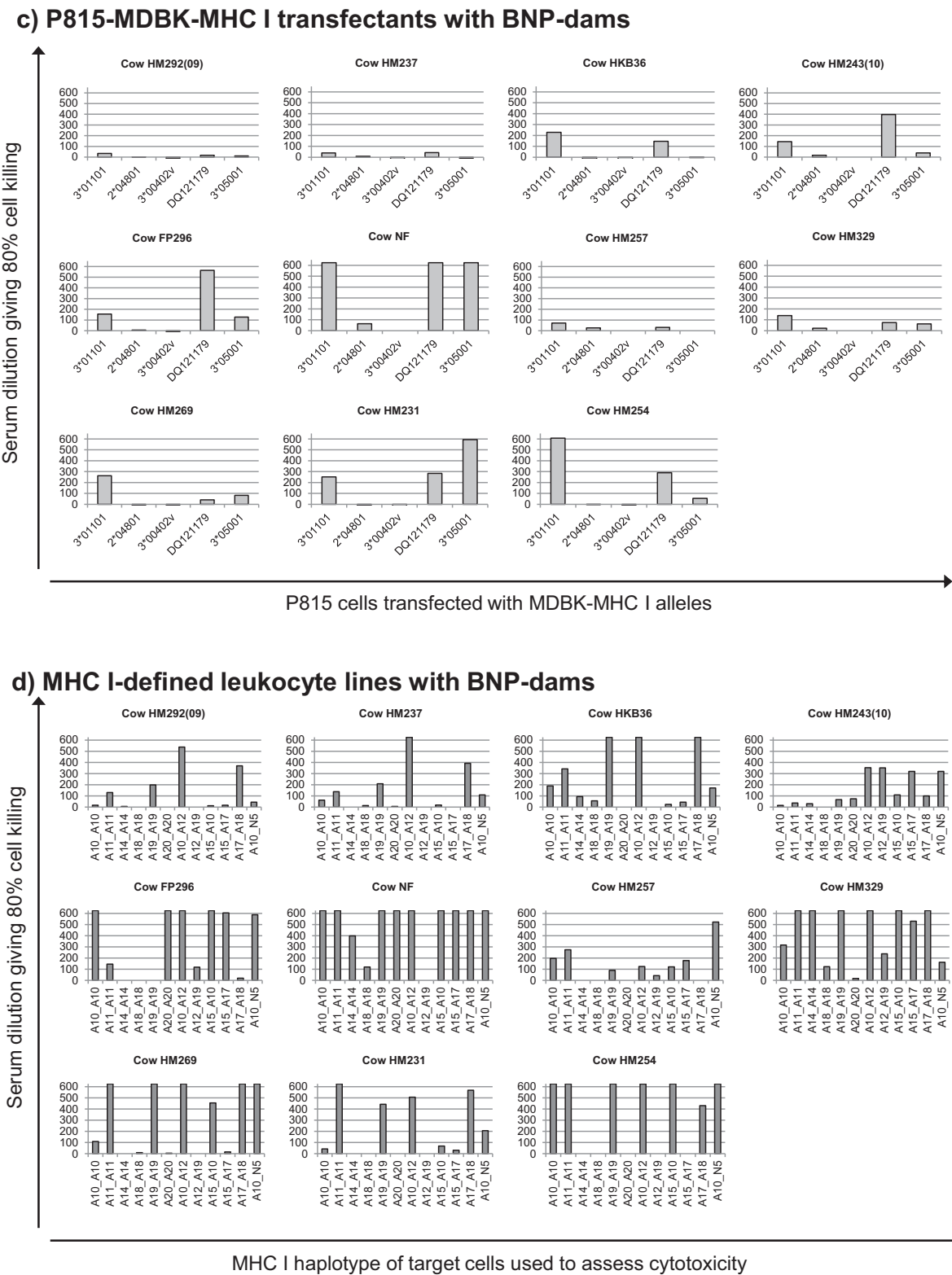
The mean PRA score, reflecting the number of cell lines recognised by the sera, was 60% for BNP-cows and 39% for those non-BNP Pregsure-cows giving a positive CDC result. There was a significant relationship between mean alloantibody titres and PRA scores from BNP-dams and Pregsure-cows (Fig. 3), with cows displaying all-reactivity against an increasing percentage of MHC I genotypes as their mean alloantibody titre increased.

Results from CDC assays performed using the MDBK-MHC I mouse transfectants or bovine leukocytes could not be compared directly due to differences in levels of MHC I expression and susceptibility to complement lysis between different cell types, and variable sharing of MDBK target epitopes by the MHC I alleles in the bovine lines (see Fig. 2). However, comparing the ranks of the



**Fig. 2.** Individual Pregsure vaccinated cows have cytotoxic alloantibodies with variable titres and specificities. Results from CDC assays with P815-MDBK-MHC I transfectants (a and c) and a panel of MHC I-defined bovine leukocyte cell lines (b and d) with serum from Pregsure-cows (a and b) and BNP-dams (c and d). Details of genes comprising the leukocyte panel can be found in Fig S1. For the panel of leukocyte cell lines, a titre of  $\geq 1/80$  giving 80% cell killing was defined as a positive reaction as no control cows or Bovilis-vaccinated cows had an alloantibody titre greater than  $1/80$ , while all BNP-dams achieved titres of  $\geq 1/80$  against at least 4 cell lines in the panel. This value is greater than previously reported results of naturally occurring pregnancy induced alloantibody in cows, where the highest titre detected by a similar method was  $1/64$  [20]. The consistently lower levels of reactivity against the P815-MDBK-MHC I transfectants compared to the leukocyte lines are attributable to consistently lower MHC I expression by the transfectants (data not shown), differences in the susceptibility of different cell types to complement lysis, and variable sharing of MDBK target epitopes by the MHC I alleles in the bovine lines.

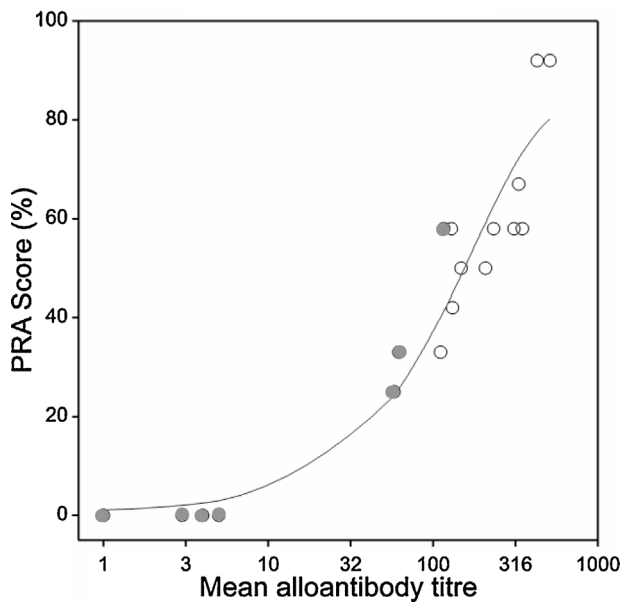




mean alloantibody titres against the panel of bovine leukocyte lines with the P815-MDBK-MHC I transfectants showed a significant correlation for BNP-dams ( $p < 0.05$ ) and Pregrese-cows ( $p < 0.05$ ) (Spearman's Rank, Genstat, VSU International)

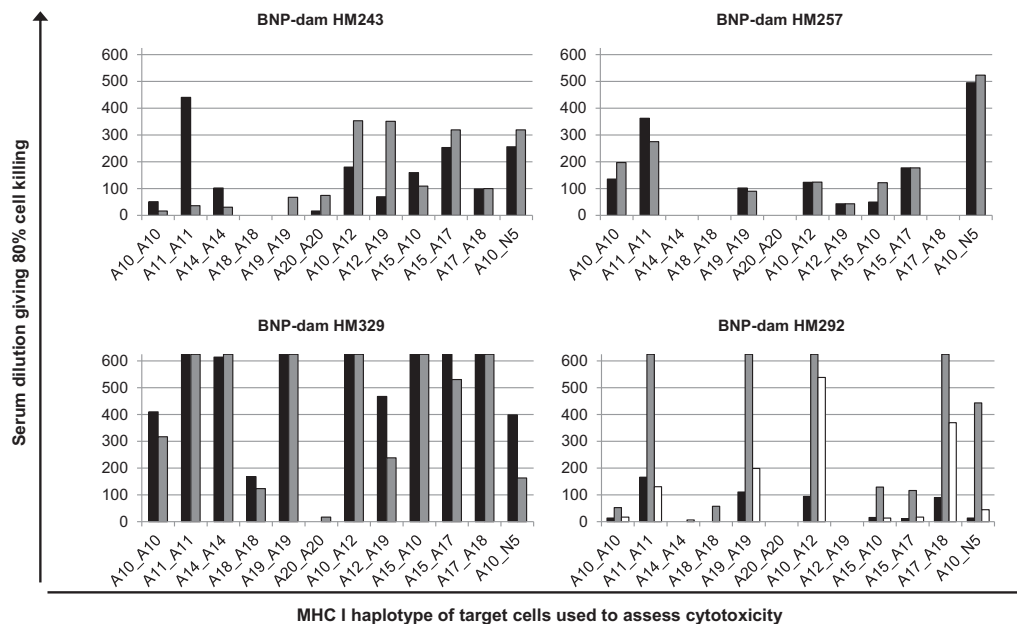
3.4. Cytotoxicity of BNP-dam sera sequential parturitions

In order to investigate whether pregnancy plays a role in the induction of BNP-alloantibody and hence the specificity of the

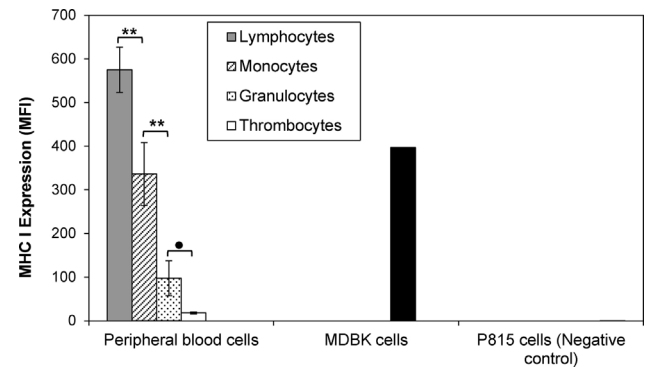


**Fig. 3.** Pregsure-vaccinated cows show allereactivity against an increasing percentage of MHC I genotypes as their mean alloantibody titre increases. Comparison of mean alloantibody titres and PRA scores from BNP-dams ( $n = 11$ ) and Pregsure-vaccinated animals ( $n = 8$ ), by fitting a non-linear regression model (sigmoid curve) (Genstat, VSU International), showed a significant relationship ( $p < 0.001$ ). The BNP-dam with the lowest PRA score and mean alloantibody titre produced a surviving BNP case, while all other BNP-dams produced fatal BNP cases. Two Pregsure-vaccinated cows had identical results (mean alloantibody titre 1, PRA score 0%) and thus only 7 data points are visible. Closed circles (●) Pregsure vaccinated dams that produced healthy calves. Open circles (○) BNP-dams.

antibodies, the cytotoxicity profile of four BNP-dams was determined at sequential parturitions, against the panel of MHC I-defined cell lines. This revealed no apparent change in specificity of the alloantibody (Fig. 4).



**Fig. 4.** The specificity of BNP-alloantibody varies little with sequential parturitions. Cytotoxicity of four BNP-dam sera at sequential parturitions (2 years for 3 cows and 3 years for 1 cow), sired by different bulls, against a panel of transformed bovine leukocyte cell lines of defined MHC I genotypes representing haplotypes expressed by >94% of the Holstein population. ■ = 2011; ▒ = 2010; □ = 2009.

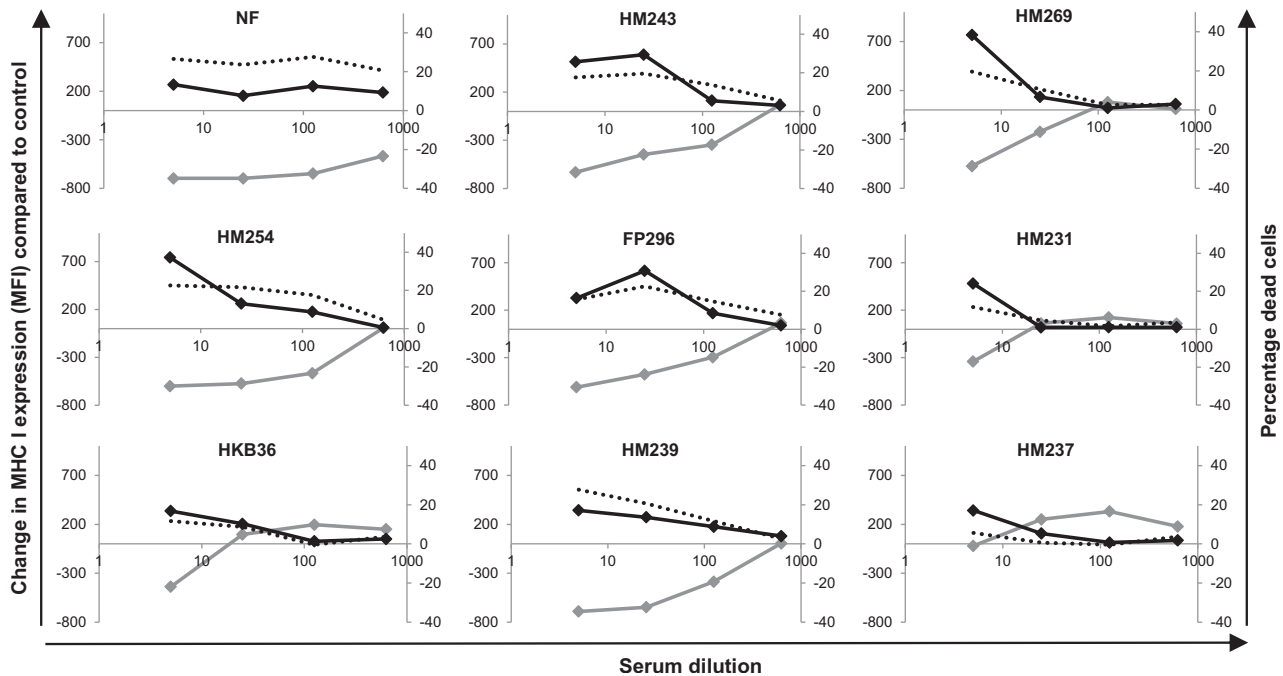


**Fig. 5.** Mononuclear leukocytes express MHC I at a significantly higher level than thrombocytes and granulocytes. MHC I expression levels, measured by median fluorescence intensity (MFI) staining with IL-A88, on bovine peripheral blood cell subtypes, MDBK cells and P815 cells. For peripheral blood cells, cell types were gated on the basis of forward and side scatter characteristics, and mean values ( $\pm$  one standard deviation) are shown. T-tests, Genstat, \*\*  $p < 0.001$ , •  $p < 0.05$ . Comparison with MDBK cells revealed similar levels of MHC I expression to that seen on monocytes.

### 3.5. Variable BNP-alloantibody damage to haematopoietic cell types

In order to investigate the hypothesis that the variation in BNP-alloantibody damage to different haematopoietic cell types is due to differences in the MHC I expression levels by the cells, three sets of experiments were performed. First, analyses of MHC I expression levels on bovine peripheral blood cells (whole leukocytes and thrombocytes) from three cows, by indirect immunofluorescence staining for MHC I and flow cytometry, showed significant differences in MHC I expression by different cell types (Fig. 5). Mononuclear leukocytes were shown to express MHC I at a significantly higher level ( $p < 0.001$ ) than thrombocytes and granulocytes.

Secondly, to assess *in vitro* cytotoxicity of BNP-sera for peripheral blood cell types, CDC assays were performed on both whole blood leukocyte and PBMC fractions obtained from three cows,



**Fig. 6.** Bone marrow cells killed by BNP-serum-induced lysis have higher MHC I expression than cells that are spared. MHC I expression, by flow cytometry, of live and dead cell populations of bovine bone marrow following BNP-serum-induced complement-mediated lysis (ILA-88+ FITC-conjugated secondary and Sytox Red). Results from 9 BNP-dams. Black line (—) dead cells, grey line (—) live cells, dotted line (...) percentage dead cells.

using sera from three BNP-dams. Significantly greater cytotoxicity ( $p < 0.001$ ) was seen for PBMC than for whole leucocytes (mean serum dilution giving 80% cell killing 1/502 for PBMC and 1/161 for leucocytes). Examination of leucocytes that survived the CDC assay by light microscopy confirmed selective killing of mononuclear cells and relative sparing of granulocytes (Fig. S2).

Thirdly, to investigate the MHC I expression levels of bone marrow cells that survived BNP-serum-induced complement-dependent lysis, unstained bone marrow cells were subjected to a CDC assay, followed by staining for MHC I expression, vital staining (Sytox Red), and flow cytometry. Analysis of the live and dead cell populations obtained showed that live cells had consistently lower MHC I expression, and killed cells consistently higher MHC I expression, compared to the control sample which had not been incubated with BNP-sera (Fig. 6). Furthermore, the percentage of dead cells and their increase in MHC I expression were significantly correlated ( $p < 0.001$ , Spearman's Rank, Genstat).

#### 4. Discussion

The establishment of a reproducible semi-automated complement-dependent cytotoxicity assay for the assessment of cow sera allowed sensitive functional quantification of the specificity of BNP alloantibody. Five expressed classical MHC I alleles were identified in the MDBK cell line, and generation of stably transfected mouse cell lines expressing these alleles allowed assessment of the specific alloreactivity of cow sera for the individual alleles. The results demonstrated that cows vaccinated with Pregsure have significantly higher titres of functional MHC I-specific alloantibodies than unvaccinated cows or cows vaccinated with an alternative BVVDV vaccine. All BNP-dams and 50% of Pregsure-cows tested were shown to have a variable profile of specificity for individual MDBK-MHC I alleles, with different cows exhibiting cytotoxicity for different alleles.

Pregsure vaccinated cows with higher alloantibody titres against the MDBK-MHC I alleles also had higher alloantibody titres against a panel of bovine leukocyte cell lines. This finding indicates

that a dominant alloantibody target on MDBK cells is shared by the leukocyte lines and, coupled with the evidence of strong reactivity with the MDBK MHC I alleles, suggests that MHC I is a major antigen. Given that none of the individual MHC I alleles identified from the MDBK cell line was present in the panel of bovine leukocyte cell lines assessed, this implies that the alloantibody measured in these experiments is cross-reactive with a number of bovine MHC I alleles. This is consistent with the nature of MHC I sequence polymorphism, where short stretches of amino acid sequence are frequently shared by different alleles. Studies of mouse monoclonal antibodies have also shown that antibodies generated against MHC I proteins expressed by a particular animal recognise a range of epitopes, which show variable cross-reactivity with other MHC I alleles [36]. Thus, it would be expected that individual Pregsure vaccinated cows produce a unique profile of MHC I-specific alloantibodies, reflecting the epitopes on their own MHC I alleles that differ from those on MDBK cells. The alloantibodies would then show varying degrees of cross-reactivity with the MHC I alleles expressed on other haplotypes. This variation, coupled with the number of these antibody specificities that are expressed by the paternal haplotype of the calf would then determine the risk of developing BNP.

Approximately 94% of Holstein cattle express at least one of the MHC I haplotypes examined in the current study [35]. Hence, based on the proportion of cell lines in the test panel that were recognised by alloantibody from individual BNP-dams, on average the sera would be expected to react with approximately 60% of calves in the Holstein cattle population, a figure that is consistent with the proportions of calves that show clinical signs of BNP in colostrum feeding experiments [5]. However, Pregsure-vaccinated cows that did not produce a BNP-calf, but had significant titres of alloantibody, would have a reduced likelihood of producing a BNP calf since they react with a lower proportion of calf MHC types as a consequence of less cross-reactive alloantibody. Thus, among Pregsure-vaccinated cows there is a continuum both of alloantibody titre and cross-reactivity of the alloantibody with other MHC I alleles, with those cows with higher alloantibody titres showing greater cross reactivity and producing clinically affected calves.



This finding is also consistent with the identification of milder subclinical forms of BNP. Those Pregsure vaccinated cows which produced alloantibody (50%) had an average PRA score of 29%, indicating an expected incidence of subclinical disease in calves of 15%. This is consistent with our recent finding that approximately 15% of calves born in a Pregsure-vaccinated herd exhibited abnormal haematological values [37].

Levels of MHC I expression on bovine leukocyte subtypes and thrombocytes were shown to correlate with the extent to which these cell types are damaged by BNP-alloantibody both *in vitro* and *in vivo* [6], with cells with higher MHC I expression levels (mononuclear leukocytes) being most susceptible to BNP-alloantibody damage. Complement-dependent killing of bovine bone marrow cells by BNP-serum was demonstrated *in vitro*, and cells surviving killing were shown to have a consistently lower levels of MHC I expression than killed cells. This provides further supporting evidence for the dominant role of MHC I-specific alloantibodies in the pathogenesis of BNP and indicates that the level of cell surface expression is a key factor in determining the selectivity of cell destruction. These findings, coupled with the complement-fixing isotype of BNP-alloantibody [38] and the kinetic similarities between lymphocyte depletion in BNP-calves and depletion using complement-fixing monoclonal antibodies [39], are consistent with a role for complement-mediated lysis in BNP pathogenesis. However further work would be required to confirm this.

Sera obtained from BNP dams at sequential parturitions showed a remarkably consistent profile of alloantibody specificities. An alteration in BNP-dam alloantibody specificity might be expected at subsequent parturitions, if BNP-alloantibody was being stimulated by exposure to foetal MHC I alleles, as naturally occurring alloantibody has broader specificity in multiparous than primiparous cows, [16]. As this is not the case, it appears the foetus does not directly influence the specificity of BNP-alloantibody. However, this finding does not preclude the possibility of the foetal calf boosting titres of pre-existing MDBK-induced specificities.

## 5. Conclusion

The results of this study provide evidence that MHC I-specific alloantibodies play a dominant role in the pathogenesis of BNP. Strong reactivity of BNP sera with the MHC I alleles expressed by MDBK cells was demonstrated. The antibody-mediated destruction of blood and bone marrow cells, measured *in vitro* by complement-mediated lysis, was shown to be dependent on the titre and specificity of alloantibody produced by individual cows and the density of surface MHC I expression by different cell types. Collectively, the results support the hypothesis that MHC I molecules originating from MDBK cells used in production of Pregsure, coupled with the use of a powerful adjuvant, are responsible for the generation of pathogenic alloantibodies.

## Contributors

Conception and design of study: CRB, NDM, TKC, WIM. Conducted experiments: CRB, KD. Data analysis: CRB, WIM. All authors read and approved the final manuscript.

## Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of this paper.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2015.05.069>

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